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Full Length Research Paper

Antioxidative potential and radioprotective ability of fermented black tea (FBT).

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This study was undertaken to evaluate the antioxidative potential and radioprotective ability of fermented black tea (FBT). Total polyphenol content in the FBT was found to be 148.30 ± 2.56 mg/g as determined by ferrous tartrate method. DPPH (2, 2-diphenyl-1-picryl hydrazyl) radical scavenging reached a maximum of 96% at 200 µg/ml of FBT and accordingly the reducing power also saturated at the very same concentration. Studies carried out with plasmid DNA revealed the ability of FBT to inhibit gamma radiation-induced single as well as double strand breaks. Prior administration of FBT to human lymphocytes significantly countered radiation-induced DNA damage as evaluated by micronuclei assay and chromosomal aberration study and cell death by trypan blue exclusion method. The results clearly indicate that FBT has significant potential to protect cellular system from radiation-induced damage and ability to scavenge free radicals might be playing an important role in its radioprotective manifestations.

Key words: Radioprotection, fermented black tea, antioxidant, free radical, CBMN assay.

INTRODUCTION

Fermented black tea (FBT) is a popular beverage among traditional fermented foods across the world, commonly known as kombucha. It is a sour beverage, prepared from the fermentation of black tea and sugar with symbiotic culture of acetic acid bacteria and yeast such as *Bacterium xylinum, Bacterium xylinoides, Bacterium gluconicum, Saccharomyces ludwigii, Saccharomyces apiculatus varieties, Schizosaccaromyces pombe, Acetobacter ketogenum, Torula varieties* and *Pichia fermentans*, which is known as „tea fungus‟ (Belloso-Morales and Hernandez-Sanchez, 2003; Ibrahim, 2013). It is composed of two portions: a floating cellulose pellicle layer and sour liquid broth (Chen and Liu, 2000). The beneficial effects of FBT are attributed to the presence of tea polyphenols, gluconic acid, glucuronic acid, lactic acid, vitamins, amino acids , antibiotics and a variety of micronutrients produced during fermentation (Vijayaraghavan et al., 2000). This beverage has been reported to have medicinal effects against metabolic diseases, arthritis, indigestion and various types of cancers (Sreeramulu et al., 2000). Recent studies have suggested that FBT prevents paracetamol induced hepatotoxicity (Pauline et al., 2001) and chromate (VI) induced oxidative stress in albino rats (Sai Ram et al., 2000). The beneficial effects of FBT such as alleviation of inflammation and arthritis, cancer prevention and immunity enhancement may be associated to its antioxidant activities.

Ionization radiation has always been part of the human environment, low linear energy transfer (LET) radiation leads to overproduction of reactive oxygen species (ROS), which induces a wide range of molecular lesions in mammalian cells that can lead to diverse cellular responses such as cell inactivation, chromosomal rearrangements and mutation, eventually resulting in cancer and hereditary diseases. DNA damage occurs, either by direct ionization or indirectly through generation of free-radical that attack DNA, resulting in single strand breaks (SSBs) and oxidative damage to sugar and base

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residues (Breen and Murphy, 1995; Von Sonntag, 1991), which may later be converted into DNA double strand breaks (DSBs).

Recently, a number of herbal extracts or their active components have been shown to render radioprotection both in *in vivo* and *in vitro* system (Weiss and Landauer, 2003; Goel et al., 1998; Kumar and Goel, 2000; Devi and Ganasoundari, 1995). Although an extensive research has been carried out to promote various beneficial health effects of FBT, its radioprotective effects have not been properly evaluated. However, Cavusoglu and Guler (2010) reported about radioprotective effect of FBT on lymphocytes on chromosomal aberration aspects only. We have already extensively studied the antioxidative and radioprotective effect of black tea against gamma radiation exposure (Pal et al., 2013; Ghosh et al., 2012). So it was of particular interest to investigate the antioxidative potential and radioprotective effect of FBT on human lymphocytes and plasmid DNA against gamma radiation induced oxidative damage.

MATERIALS AND METHODS

Chemicals

DPPH (2, 2-diphenyl-1-picryl hydrazyl), pBR322 DNA, colcemid, histopaque (HP) 1077, and cytochalasin-B were obtained from Sigma-Aldrich (St Louis, MO). Potassium ferricyanide, ferrous sulfate, trichloroacetic acid (TCA), potassium sodium tartrate tetrahydrate, ferric chloride and Roswell Park Memorial Institute (RPMI) 1640 media were purchased from Himedia, Mumbai, India. Fetal bovine serum (FBS), phytohemagglutinin (PHA) and penicillin-streptomycin were obtained from Gibco, Grand Island, NY. All reagents used were also of analytical grade and were procured from local distributors.

Spectrophotometric analysis

All the spectrophotometric analyses were performed at room temperature using matched quartz cells of 1 cm path length with the help of Varian UV-visible spectrophotometer (CARY 100 Bio, USA).

Irradiation

Gamma-irradiation was carried out using ^{60}Co Gamma Chamber 1200 (BRIT, India) at University Grant Commission-Department of Atomic Energy (UGC-DAE) Centre for Scientific Research, Kolkata. The dose rate was 3.96 kGy/h.

Preparation of FBT

The black tea extract was prepared by adding black tea leaves (Lipton Darjeeling Tea, 2% w/v) and Sucrose (10%, w/v) in boiled water and allowed to infuse for about 15 min. The infusion was then filtered through sterile sieve and the preparation was allowed to cool at room temperature. 200 ml of black tea extract was poured into 500 ml glass jar that had been previously sterilized and inoculated with 3% (w/v) of freshly grown tea-fungus that had been cultured in the same medium for 7 days and 10% (v/v) of previously fermented liquid tea broth aseptically. The jar was carefully covered with a clean cloth and locked properly. The fermentation was carried out in a dark incubator at 25°C for 5-6 days. Later, the culture was centrifuged at 5000 rpm for 30 min aseptically and used for experiments. There was found no evidence of contamination in fermented products (Ibrahim, 2013).

HPLC profile of FBT

Components of FBT were identified by Waters HPLC system using reverse phase C18 column (3.9 × 150 mm, 4 µm) using mobile phase composed of methanol/water (50:50 v/v, pH 2.5 with trifluoroacetic acid) at 365 nm for identification of flavonols. Tea flavonoids at pure form were used for identification. Detection was performed by Waters 486 Tunable Absorbance Detector at the flow rate of 1 ml/min. Data analysis was performed by Empower 2 software.

Determination of total polyphenol content

The total polyphenol content was determined by the ferrous tartrate method (Pal et al., 2012; Turkmen et al., 2006). Absorbance of test sample was recorded at 540 nm using a blank solution prepared with distilled water replacing FBT. The content of tea polyphenols was calculated by the following equation:

$$\text{Polyphenol content (mg/g)} = (E_1 - E_2) \times 3.9133 \times 50/1$$

where, E_1 = Absorbance of the tested solution at 540 nm; E_2 = Absorbance of the control solution at 540 nm; 3.9133 = Constant (polyphenol concentration was 3.9133 mg/ml when absorbance at 540 nm was 1.0); 50/1 = Constant (1 g of tea sample was extracted in 50 ml water).

DPPH scavenging ability

The scavenging ability of the samples on DPPH radical was determined according to the method as reported by Pal et al. (2013) with little modification. Briefly, 50 µl of FBT diluted fifteen fold with distilled water and mixed with an aliquot of 1950 µl of 0.06 mM DPPH radical in methanol. The reaction mixture was vortex-mixed and allowed to stand at 25°C In the dark for 30 min. Absorbance at 517 nm was measured using methanol as a blank. Antioxidant activity was expressed as percentage

inhibition of the DPPH radical and was determined by the following equation:

Antioxidant activity (%) = (Absorbance $_{control}$ – Absorbance $_{sample}$) / Absorbance $_{control}$ × 100

Reducing potential

In a test solution, 20 µl of FBT was mixed with 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The reaction mixture was incubated at 50°C for 30 min. To this solution, 2.5 ml of 10% TCA was added and centrifuged for 10 min at 3000 rpm. From the supernatant, 2.5 ml was taken and mixed with 2.5 ml of distilled water and 0.5 ml of 0.1% ferric chloride. Absorbance of final mixture was measured at 700 nm. Higher absorbance value of the solution indicated higher reducing power (Yildirim et al., 2001; Pal et al., 2013).

Plasmid DNA damage study

Effect of FBT on radiation-induced DNA strand breaks was studied on plasmid pBR322 DNA as described by Thibodeau et al. (2001). In this method, 250 ng of plasmid DNA in 50 mM phosphate buffer solution (PBS) of pH 7.4 was irradiated with a standardized dose of 50 Gy, which was considered sufficient to convert all supercoiled (sc) DNA into open circular (oc) form. Plasmid DNA was irradiated in the absence and presence of FBT of concentrations 10-100 µg/ml. After irradiation, the DNA was subjected to electrophoresis in 1% agarose gel for 20 min at 90 V by using 0.8 mM Tris borate/2 mM EDTA buffer (pH 8.3), at room temperature and stained with ethidium bromide (0.5 µg/ml). The ethidium bromide-stained DNA bands were photographed and analyzed by Bio-Rad Gel Doc system. The bands were quantified by densitometry scanning of negative prints with the software provided with the system.

Cell viability assay

Cell viability assay was carried out by trypan blue dye exclusion method (Ghosh et al. 2012). Briefly, freshly isolated normal lymphocytes from healthy donors were treated with or without FBT (2-20 µg/ml) and incubated for 30 min at 37°C incubator. After washing the cells twice in PBS, they were exposed to 4 Gy of gamma radiation and incubated for 24 h. After incubation, 10 µl trypan blue was mixed with same volume of cell suspension and kept for 5 min. Cell viability was measured by Countess Automated Cell Counter (C10277, Invitrogen, CA).

Cytokinesis-block micronucleus (CBMN) assay

Lymphocytes were isolated from the blood using HP 1077 according to the method described by Boyum (1974). The solution of lyophilized FBT was filtered using 0.22 µm syringe filter. FBT was then added to the lymphocyte suspension in RPMI 1640 media to achieve final concentrations of 2-20 µg/ml. Samples were incubated for 1 h at 37°C in humidified atmosphere of 5% CO_2 in air and after washing twice in PBS, the cells were subsequently exposed to 4 Gy of radiation (Rao et al., 2006; Tiwari et al., 2009).

After irradiation, cell culture was setup with 5 ml of RPMI 1640 media supplemented with 10% FBS at 37°C in a CO_2 incubator. Cytochalasin B (6 µg/ml) was added to the cultures at 44 h. After 72 h, lymphocytes were harvested after short treatment with 75 mM of KCl and immediately fixed in a mixture of methanol and glacial acetic acid (3:1). After that, cells were spread on clean slides and stained with 2% Geimsa in Sorenson's buffer (pH 6.8) for 10-12 min. All slides were coded and minimum of 1000 cells were evaluated for the frequency of micronuclei in cytokinesis-blocked binucleated cells. The micronuclei were identified and scored by a single person according to the criteria of Fenech et al. (2003) and expressed as percentage of micronucleated binucleate cells (MNBNC%).

Chromosomal aberration study

Lymphocytes were isolated and treated with FBT prior to irradiation as described above. Immediately after 4 Gy of irradiation, 1 ml of irradiated lymphocytes was suspended in 8 ml of RPMI 1640 medium supplemented with 20% (v/v) FBS, 300 U/ml penicillin-streptomycin, 90 µg/ml PHA and the culture was incubated at 37°C in CO_2 incubator for 72 h. At the 71st h, 0.05 µg/ml colcemid was added to the medium. After 72 h incubation, the cultured cells were treated with hypotonic 0.56% KCl solution for 15 min and subsequently fixed in freshly prepared acetic acid-methanol (1:3) solution. The fixative was changed at least three times before the cell suspension was dropped onto pre-cleaned chilled microscopic glass slide and dried at room temperature at least for two days (Pathak et al., 2007). Giemsa staining method was applied for scoring metaphase chromosomes. Chromosomal aberrations were scored under a binocular light microscope (Carl ZEISS, Germany) according to Savage"s classification (Savage, 1976).

Statistical analysis

The statistical analysis of the samples was undertaken using Student's t-test. All data reported are means ± SD for three independent experiments, unless otherwise noted. The data were analyzed by Origin software (Version 8) for Windows.

RESULTS

HPLC profile of FBT

The HPLC profile of FBT using mobile phase methanol/water (50: 50 v/v, pH 2.5 with trifluoroacetic

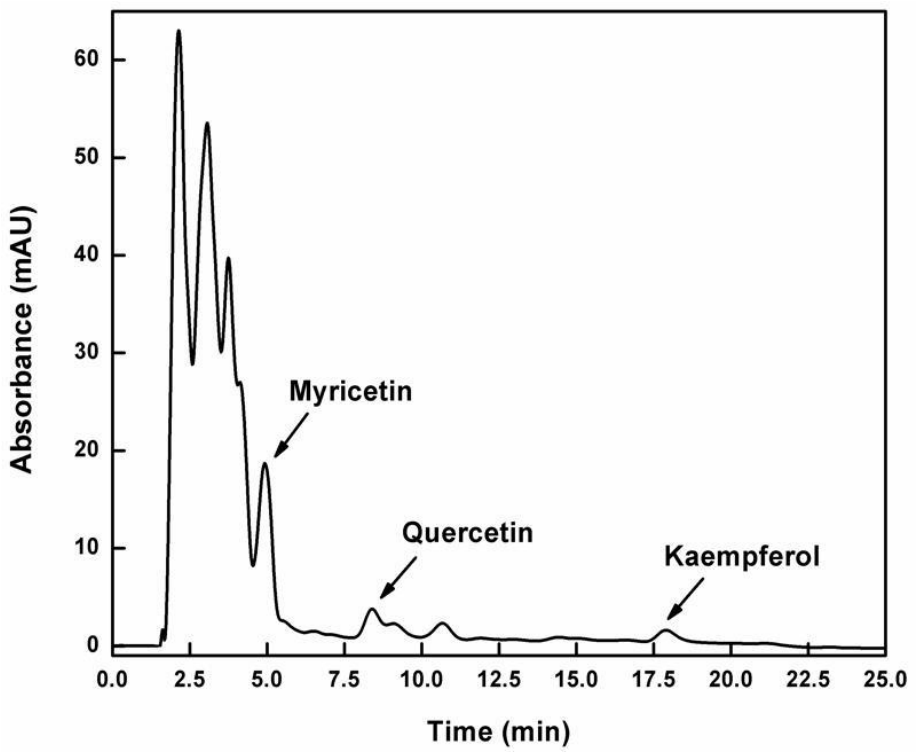

Figure 1. Analysis of polyphenols in FBT by HPLC. Chromatograms of FBT in a mobile phase consisting of methanol/water (50:50 v/v, pH 2.5 with trifluoroacetic acid) at 365 nm for identification of flavonols.

acid) at 365 nm revealed seven clearly identifiable peaks as shown in Figure 1. The three peaks shown in chromatogram are identified as myricetin, quercetin, and kaempferol using pure forms as standards.

pH analysis

The pH of the sample was measured with an electronic pH meter (Eutech Instruments, USA). The pH of the FBT used for this experiment, that is, after 5-6 days of incubation was found to be 3.8 ± 0.6.

Determination of total polyphenol content

Total polyphenol content in the FBT was found to be 148.30 ± 2.56 mg/g as determined by ferrous tartrate method.

DPPH scavenging ability

Increasing concentration of FBT significantly scavenge DPPH radical in a dose dependent manner and saturated (about 96%) at a concentration of 200 µg/ml onwards (Figure 2).

Reducing potential

The reducing potential of FBT showed a dose dependent increase, with increasing concentration as shown in Figure 3. Saturation in reduction of ferric complex in the reaction mixture was observed at 200 µg/ml onwards.

Plasmid DNA damage study

Exposure to gamma radiation leads to DNA strands breaks in pBR322, resulting in relaxation of plasmid DNA from super-coiled (sc) form to open circle (oc) form. The changes in the relaxation process rendered by FBT of various concentrations (10-100 µg/ml) when exposed to a dose of 50 Gy radiation are shown in Figure 4.

Cell viability assay

For this assay, 4 Gy gamma radiation-induced significant ($p < 0.05$) cell killing and surviving or viable fraction was reduced to 51.51 ± 2.21% considering untreated control as 100% (Figure 5). Pre-irradiation administration of increasing concentrations (2-20 µg/ml) of FBT significantly increased cell viability and maximum cell survival (86.04 ± 2.11%) was observed at 5 µg/ml. However, FBT alone at all possible experimental concentrations did not show any toxic effects on cells (data not shown in figure).

CBMN assay

Treating lymphocytes with various concentrations of FBT

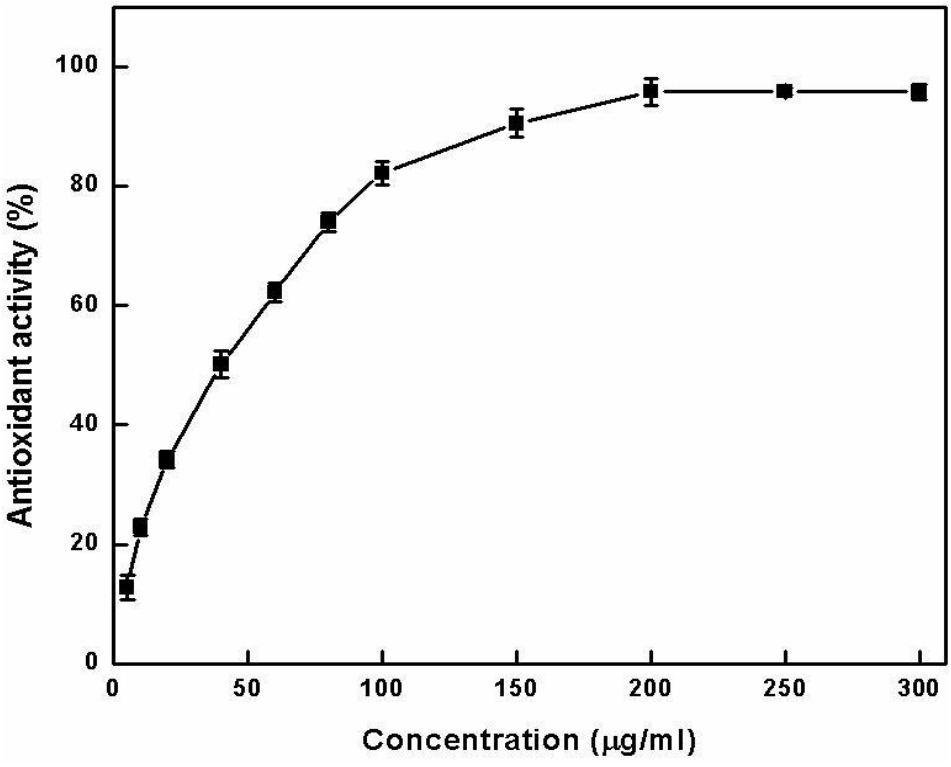

Figure 2. Plot of percentage (%) of scavenging activity on free radical 2, 2-diphenyl-1-picrylhydrazyl (DPPH) of different concentrations (5-300 µg/ml) of FBT. Error bars indicate the standard deviation for 4 independent experiments.

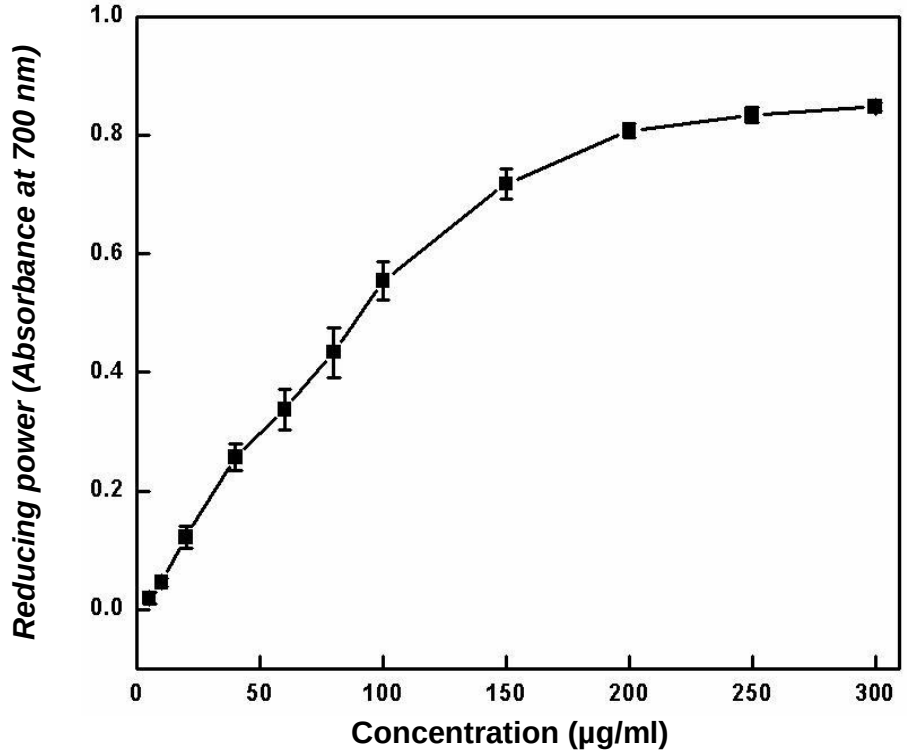

Figure 3. Plot of absorbance at 700 nm versus increase in FBT concentration (5-300 µg/ml) to evaluate reducing power FBT.

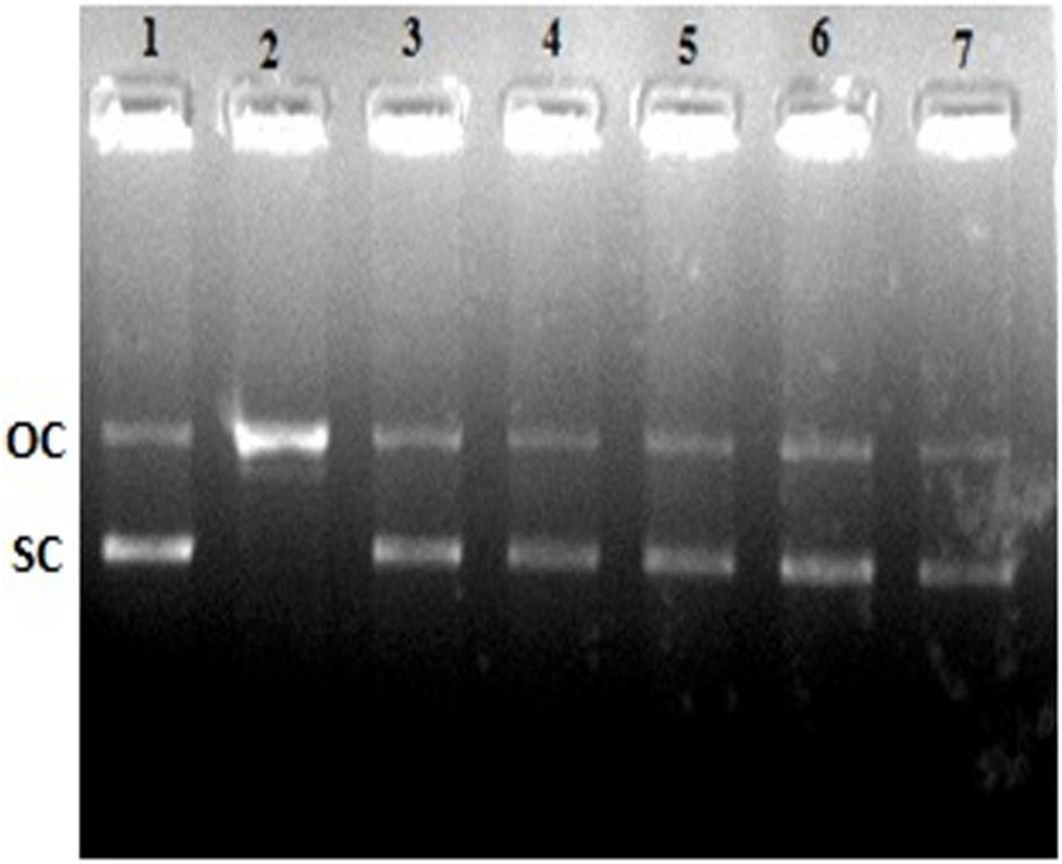

Figure 4. Effect of varied concentrations of FBT (10–100 µg/ml) on gamma radiation-induced DNA strand breaks in pBR322 plasmid DNA. OC = open chain, SC = super coiled. Lane 1: Control; Lane 2: 50 Gy radiation (R); Lane 3 to 7: increasing concentration (10-100 µg/ml) of FBT + 50 Gy radiation.

(2, 5, 10 and 20 µg/ml) for 1 h before exposure to 4 Gy of gamma radiation resulted in a significant decrease ($p < 0.001$) in the percentage of micronucleated cells, that is, MNBNC to 34, 17, 26, and 32%, respectively, when compared with the radiation-alone group (44%) as shown in Figure 6.

Chromosomal aberration study

The distribution of different types of chromosomal aberrations in human peripheral lymphocytes is shown in Table 1. The table shows the status of mitotic chromosomal aberration when subjected to 4 Gy of gamma radiation and pre-treatment with various concentrations (2-20 µg/ml) of FBT before irradiation. According to Savage‟s classification, six structural chromosomal aberrations were determined in the control and experimental groups.

DISCUSSION

Since the radioprotective effects of synthetic chemicals (for example, WR-2721) are short term and associated with severe side effects such as vomiting, nausea, diarrhea, hypotension, hypocalcaemia, nephro and neuro-toxicity at clinically effective doses (Cairnie, 1983; Glover et al., 1983; Kligerman et al., 1984; Landauer et al., 1987), the use of good natural radioprotector represents an obvious strategy to improve the therapeutic index in radiotherapy. We have already established black tea (*Camellia sinensis*) extract as a potent antioxidant and a probable radioprotector in our previous work (Pal et al., 2013). The present study was aimed at understanding the role of the fermented variety of black tea in ameliorating gamma radiation induced oxidative damage under *in vitro* condition.

Thorough investigation of antioxidant status of FBT revealed that it significantly scavenged DPPH radical with increasing concentrations as shown in Figure 2. At a dose of 200 µg/ml, the scavenging ability saturates which is about 96%. This scavenging ability may be attributed to the presence of phenolic hydroxyl groups in FBT. The antioxidant activity of natural compounds is known to have a direct correlation with their power to act as reducing agent. FBT was found to reduce Fe^{+3} to Fe^{+2} and maximum reduction was achieved at 250 µg/ml

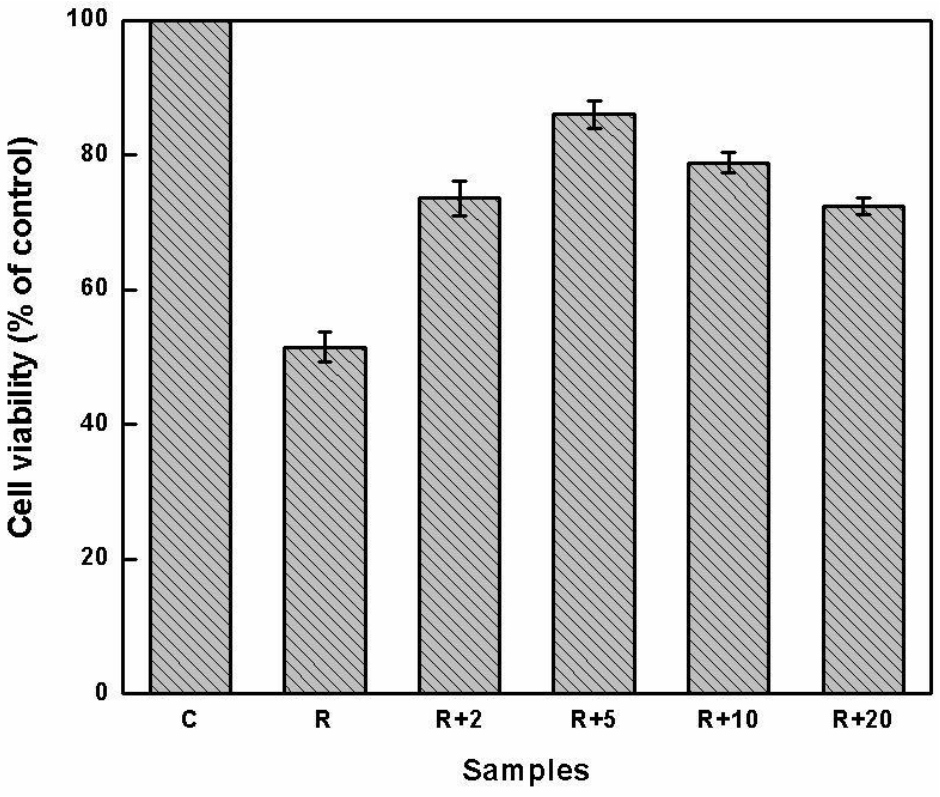

Figure 5. Effect of varied concentrations of FBT (2-20 µg/ml) on 4 Gy of gamma radiation (R) induced change in cell viability (%) of viable normal lymphocytes. Error bars indicate the standard deviation for 4 independent experiments.

onwards as evident from Figure 3. Reduction power of a compound is related to the electron transfer ability of compounds like quercetin, myricetin and kaempferol, the presence of which is confirmed by HPLC analysis (Figure 1).

Exposure of lymphocytes to 4 Gy gamma radiation resulted in a significant reduction in cell survival as measured by trypan blue dye exclusion assay (Figure 5). This could be attributed to generation of reactive oxygen species (ROS) and oxidation of critical biomacromolecules (Ross, 1999). However, pre-irradiation treatment of FBT resulted in significant reduction in radiation-induced cell killing clearly establishing its protective efficacy.

DNA being the major site of radiation-induced damage, the capacity of FBT to prevent DNA damage was investigated using plasmid DNA pBR322. Induction of breaks in plasmid DNA resulted in conversion of supercoiled (sc) form into slow migrating open chain (oc) relaxed form or linear form. This simple assay has been widely used to study effects of various pro and antioxidant properties of a number of compounds (Thibodeau et al., 2001). In corroboration with cell survival, FBT inhibited radiation-induced strand breaks in plasmid DNA. From the electrophoresis profile (Figure 4), the result indicates that most of the sc form of plasmid DNA is converted into oc form, when irradiated at 50 Gy, and sc from getting protected upon treatment with FBT (10-100 µg/ml) before irradiation.

It is well established that ionizing radiation induces different types of lesions in DNA including SSBs, DSBs, base damage as well as DNA cross-links. Among all these, DSBs have been considered the critical lesion for the radiation-induced chromosome break and cell death (Natarajan et al., 1986; Iliakis, 1991). There was a correlation established between the induction of cell death, chromosome aberrations and the frequency of micronuclei formation (Midander and Revesz, 1980). The genotoxicity or cytogenetic damage induced by radiation on the mitotic cell is expressed as an increase in micronuclei frequency during the interphase after the first post-treatment mitosis. Moreover, it is understood that non-repair or mispair of DNA DSBs contributes to the chromosomal aberrations (Natarajan et al., 1986; Bryant and Iliakis, 1984), which could be analyzed by the quantitative analysis of micronuclei. Therefore, CBMN assay is a very useful parameter for assaying cytogenetic damage and is extensively used to screen the radio-ameliorating potential of synthetic and natural products (Jagetia and Venkatesha, 2005 ; Pal et al., 2013). Our

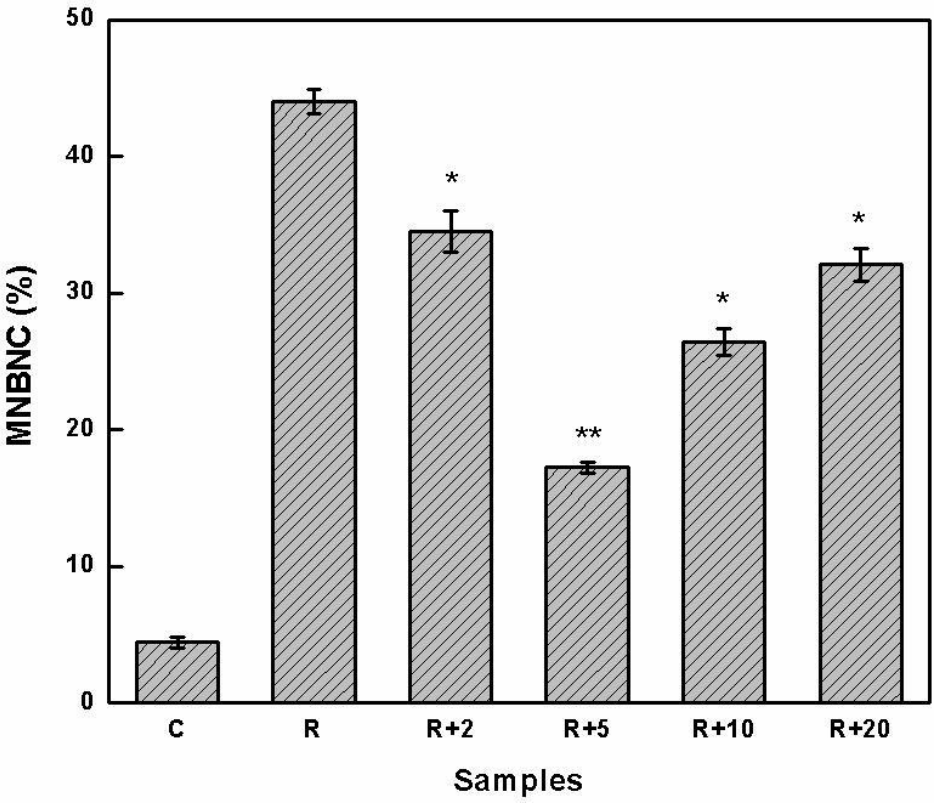

Figure 6. Effect of varied concentrations of FBT (2-20 µg/ml) on 4 Gy of gamma radiation (R) induced cellular DNA damage in normal lymphocytes. MNBNC% = percentage of micronucleated binucleate cells (*p < 0.005, **p < 0.0001 as compared to radiation-alone group).

Table 1. Distribution of varied concentrations of FBT (2–20 µg/ml) on 4 Gy of gamma radiation (R) induced chromosomal abnormality in normal human lymphocyte cells.

Sample	Acentric	Dicentric	Ring	Break	Fragments	Gap
Control	2.34±1.53	0.00±0.00	0.00±00	2.67±1.15	3.6±1.15	0.00±0.00
R	26.00±3.00	19.00±2.00	12.34±1.24	33.34±1.53	39.6±2.08	9.00±2.00
R+2 µg/ml	20.00±2.64	14.00±1.00	9.00±0.81	23.34±2.08	32.00±2.64	7.00±1.00
R+5 µg/ml	12.67±2.08	9.33±1.52	2.67±1.24	13.00±1.00	15.30±1.78	1.70±1.53
R+10 µg/ml	15.67±2.51	11.00±1.73	4.00±0.81	16.67±0.57	19.30±1.52	3.00±2.00
R+20 µg/ml	19.00±2.00	12.67±2.08	7.00±1.63	18.67±1.52	24.6±0.57	5.30±1.15

studies demonstrated that FBT was effective in counteracting the genotoxic effect of gamma radiation and thereby offering radioprotection. FBT alone was non-toxic to the lymphocytes in all the tested doses. It is interesting to note that FBT was very effective in ameliorating the effect of radiation at dose of 5 µg/ml. Moreover, a significant protection window was observed for the range of radiation doses (Figure 6). Chromosomal aberrations assessment is another very important tool to monitor genotoxic effects due to irradiation. The experimental data (Table 1) obtained in the present study revealed that the frequency of chromosomal aberration parameters such as breaks, gaps, fragments, etc., were increased when irradiated with 4 Gy gamma radiation. However, reduction in frequency of these parameters upon treatment with FBT before irradiation clearly established FBT as a potential radioprotector.

This study reports for the first time the radioprotective potential of FBT *in vitro*. Although, at this stage, the exact mechanism of action of FBT on radioprotection is far from being understood, the available information on the antioxidant potential of FBT obtained from the present

investigation supports the free radical scavenging mediated pathways.

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